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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Derek van der Kooy et al.	Art Unit:	1636
Serial No.:	09/333,248	Examiner:	R. Yucel
Filed:	June 15, 1999	Customer No.:	21559
Title:	PHARMACEUTICALS CONTAINING RETINAL STEM CELLS		

Assistant Commissioner for Patents
Washington, DC 20231

DECLARATION OF DR. VINCENT TROPEPE UNDER 37 CFR §1.132
TRAVERSING GROUNDS OF REJECTION

Under 37 C.F.R. §1.132 and regarding the rejection of claims 5-8 under 35 U.S.C. § 112, first paragraph, for lack of enablement, I declare:

1. I am a named inventor of the subject matter claimed in United States Patent Application Serial No. 09/333,248 filed on June 15, 1999.

2. I attended the University of Toronto where I obtained a Ph.D. in Developmental Biology. I am currently a post-doctoral fellow at the Massachusetts Institute of Technology. My education and professional experience are described in further detail in my curriculum vitae, a copy of which is attached as Appendix A.

3. I have read and understood the Office Action, dated April 11, 2001. This Declaration is presented to overcome the rejection of claims 5-8 under 35 U.S.C. § 112, first paragraph, for lack of enablement.

4. The methods of the invention have been used by researchers working under the direction of both myself and Dr. van der Kooy, also an inventor on the above-referenced application, to successfully and reliably demonstrate intraocular transplantation of retinal stem cells and retinal stem cell-derived cells, and to demonstrate that these cells have the potential for differentiating into mature retinal cells.

5. The materials and methods employed were known to those skilled in the art of cellular transplantation at the time the application was filed or are provided by the instant specification and were as follows:

Procedure for preparing adult mouse retinal stem cell-derived cells for transplantation.

Adult mice carrying the green fluorescent protein (GFP) transgene were used as a source of donor tissue. The eyes of these mice were used to isolate retinal stem cells according to the procedure outlined in the instant specification (see, e.g., page 20, line 21, through page 23, line 11). After 7 days *in vitro*, verification that the retinal spheres expressed significant levels of the GFP transgene was determined by using an inverted fluorescence microscope. Retinal spheres expressing the transgene appeared uniformly fluorescent. These spheres were then dissociated into a single cell suspension (see page 21, line 22, through page 23, line 11) and resuspended in Hank's phosphate buffered saline (PBS). Cell concentration and viability was quantified using the trypan blue exclusion method.

Procedure for transplanting adult mouse retinal stem cell-derived cells into newborn mouse eyes.

The dissociated cell suspension was loaded into a Hamilton microsyringe using a 25G 5/8" needle at a concentration of 10,000 cells/0.5 µl. Newborn CD1 mouse pups (not older than 24 hours) were used as recipients for the cell transplants. The animals were anaesthetized using Halothane (inhalant). A small guide hole was made through the skin of the eyelid (previously sterilized with 70% ethanol) at the lateral margin of the right eye using a 25G 5/8" needle. Using the Hamilton microsyringe loaded with the cell suspension, 0.5 µl of the suspension was slowly injected (over a period of 10-20 sec) into the vitreous. Immediately after the solution was

injected, the syringe was held in place for another 10-20 sec before it was slowly removed. The injection site was cleaned and sterilized again with 70% ethanol before the pup was returned to the mother's nest in the homecage. A total of 32 pups were used for these experiments. Using this method, bleeding was practically non-existent and surface wounds were very quickly healed to the point that both the injected (right) and non-injected (left) eyes were indistinguishable by the end of the transplanting session. The transplant recipients were allowed to survive with their littermates for 4 weeks. In that time, there were no overt signs of infection or deformity in the injected eyes. Food and water consumption was normal for CD1 mice and daily activity (e.g. grooming) appeared normal.

Procedure for assessing transplanted cells.

After a 4-week survival period, animals were killed with an anaesthetic overdose and transcardially perfused first with 0.9% saline and subsequently with 4% paraformaldehyde in a PBS solution. Eyes were removed by transecting the optic nerve and post-fixed in 4% paraformaldehyde at 4°C overnight. The eyes were then transferred to a cryprotectant solution containing 20% sucrose for at least 24 hours at 4°C. Subsequent to cryoprotection, eyes were flash-frozen with CO_2 and maintained at –20°C while cutting 14 μm sections on a cryostat. Sections from injected and non-injected eyes were processed using immunocytochemistry to determine the position and morphology of the transplanted cells and to determine whether the transplanted cells differentiated and expressed protein "markers" of mature retinal cells. Primary antibodies used to detect markers of mature retinal cells included Rho4D2, B630, 7H2 and D2P4 (to detect mature rod photoreceptors), βIII-tubulin (to detect retinal ganglion cells), and 10E4 and GFAP (to detect Müller glial cells). Given that the donor cells express GFP, the secondary antibodies used in each case were covalently linked to either rhodamine (TRITC) or cyanine (CY3) fluorophores with emission spectra (red) easily distinguished from GFP with appropriate filters. The sections were then viewed on either an inverted fluorescence microscope or a confocal microscope.

6. The results of the experiments described in paragraph 5 were as follows:

Extensive integration of GFP+ donor cells within the host retina after a 4-week survival period was observed. Donor cells were detected within several layers of the multi-layered retina. For instance, GFP+ cells were observed in the area of the inner nuclear layer juxtaposed with the inner and outer plexiform layers. Expression of the Müller glial cell marker 10E4 (which labels glial cells extending throughout the entire extent of the retina) appears to correlate with a small fraction of the donor cells in the inner nuclear layer. The vast majority of donor cells, however, appeared to migrate through the inner cell layers of the retina (ganglion cell layer, and inner nuclear layer) and settle in the outer nuclear layer of the retina (photoreceptor cell layer) (Figure 1, a copy of which is attached as Appendix B). The GFP+ cells in the outer nuclear layer resembled rod photoreceptors in morphology and expressed several markers specific for mature rods. For instance, the rod marker Rho4D2 corresponded to the position of the outer segment of the rod cells and was co-expressed with GFP in these outer segments (see Figure 1, Panel B). Other rod markers (B630, 7H2, D2P4) were similarly co-expressed in GFP+ cells in this region. In some instances, the extent of integration of these cells in the photoreceptor layer was significant, yet layer morphology was largely undisrupted. Thus, these data suggest that a large number of retinal stem cell-derived cells differentiate into mature rod photoreceptors with normal morphological and biochemical properties after transplantation.

7. The data described in paragraph 6 clearly demonstrate the successful intraocular transplantation of retinal stem cell-derived cells. The retinal stem cells differentiate into retinal neurons, in particular, photoreceptors. These data were generated using morphological and immunocytochemical techniques described in the instant specification on page 24, line 9, through page 25, line 10.

8. The recipient mice of the experiments described in paragraphs 5 and 6 were healthy and active and microscopic examination of recipient retinae revealed no abnormal retinal development or adverse immune response.

9. Successful transplantation of retinal stem cells, in the absence of an adverse immune reaction, has also been demonstrated recently by Kurimoto et al. (Soc. Neurosci. Abstr., vol. 31, Program No. 791.11, 2001; provided herewith as Appendix C). Kurimoto et al. demonstrate that retinal stem cells can differentiate into photoreceptor-specific cell types post-transplantation into the retina.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Respectfully submitted,

Date: 4.11.02

Vincent Tropepe

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